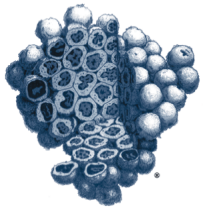

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Circulating Connective Tissue Precursors: Extreme Rarity in Humans and Chondrogenic Potential in Guinea Pigs

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Key words. blood cells • cell culture • cell transplantation • chondrogenesis

ABSTRACT

Using a variety of cell separation techniques and cultivation conditions, circulating, adherent, connective tissue, clonogenic cells were found in just 3 donors out of 66 demonstrating that these precursors are extremely rare in postnatal human blood. Contrary to humans, guinea pig blood shows much more reproducible connective tissue colony formation; it was therefore chosen to study the differentiation potential of adherent blood-derived clonogenic cells. Out of 22 single colony-derived strains of various morphologies, only 5 spindle-shaped strains showed extensive proliferative capacity *in vitro*. None of these strains formed bone upon *in vivo* transplantation, whereas two strains formed cartilage in high-density pellet cultures *in vitro*. Both chondrogenic strains included cells expressing aggrecan, while non-chondrogenic strains did not. Out of 4 polyclonal strains studied, one formed both cartilage and abundant bone accompanied by hematopoiesis-supporting stroma. Evidently, there are cells in adult guinea pig blood capable of both extensive proliferation and differentiation towards cartilage: circulating chondrogenic precursors. While some of these cells lack osteogenic potential and therefore represent committed chondrogenic precursors, others may be multipotential and consequently belong to the family of skeletal stem cells. This is the first demonstration of postnatal circulating chondrogenic precursors, as well as of precursor cells with chondrogenic but not osteogenic potential.

INTRODUCTION

Transformation of blood leukocytes into connective tissue cells belongs to the oldest and most controversial concepts in cell biology. One of the highly contentious issues within this concept is the interpretation of human peripheral blood cultures where macrophages and their derivatives can acquire notoriously diverse morphological and tinctorial phenotypes often displaying a transitory fibroblast-like morphology.[1, 2] Consequently subsets of these cells have frequently been mistakenly identified as fibroblast-like cells,[3-7] thus incorrectly suggesting an abundance of adherent connective tissue progenitors in human blood. However, when rigorous criteria are applied, including precise immunophenotypying, the ability to form discrete, compact colonies[8] and to undergo multiple passages generating fibroblast-like

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strains,[9] the capacity for connective tissue formation upon *in vivo* transplantation in diffusion chambers[10-13] or for bone formation upon *in vivo* transplantation in an open system,[14] true adherent connective tissue precursors are either totally absent[2, 15-17] or very rare[14] in adult human blood. In the first part of this study, we used a variety of cell separation techniques and cultivation conditions to establish blood cell cultures from a significantly extended and diversified group of human donors. Despite all the variables, adherent colony formation was observed in cultures from just 3 donors out of 66 demonstrating that circulating connective tissue precursors are absent in the vast majority of human donors, and extremely scarce in the remaining few.

Unlike humans, blood cells of several animal species, such as mouse, rabbit, and, in particular, guinea pig, show much more reproducible formation of adherent connective tissue colonies.[8, 14, 18, 19] In the second part of the study, the guinea pig model was chosen to further analyze the differentiation potential of adherent strains of blood-derived cells in a search for circulating progenitors of solid-phase connective tissues. It was shown that some single colony-derived and polyclonal strains of circulating guinea pig cells differentiated to form cartilage, thus demonstrating for the first time the existence of postnatal circulating chondrogenic precursors.

METHODS

Human model

Human blood specimens

Human blood specimens were obtained with informed consent per institutionally approved protocols. Peripheral blood from 66 donors was received either as whole blood (34 donors) or as one of the following blood products provided by the Blood Services Section, Division of Transfusion Medicine, Clinical Center, NIH: buffy coat, or total leukocyte fraction (28), leukoapheresis product, or leukocytes minus granulocytes (3), monocyte fraction (1). Blood was received from 39 males and 27 females of the following age groups: 0 to 9 y.o. – 7 (including one newborn, three children from 4 to 6, and three children from 7 to 9 years old); 10 to 19 y.o. – 7; 20 to 29 y.o. – 7; 30 to 39 y.o. – 10; 40 to 49 y.o. – 14; 50 to 59 y.o. – 11; 60 to 69 y.o. – 5; 70 y.o. and older – 5. Thirty seven donors were healthy volunteers (including one who received G-CSF injections at 10 μg/kg for 5 days); from 19 patients, blood was taken within several hours after they had suffered fractures of one or more bones; 4 patients had fibrous dysplasia of bone or McCune-Albright syndrome (OMIM#174800); 1 had fibrodysplasia ossificans progressiva (OMIM #135100); 4 had juvenile dermatomyositis with calcinosis; 1 had oncogenic osteomalacia. Most cultures were established within several hours after blood collection, with the exception of trauma patients whose blood was sent overnight so that the cultures were prepared on the next day. The total number of nucleated cells ranged from 11.5 x 10^6 to 1,690 x 10^6 per blood specimen.

Blood cell preparation

Blood cells were plated either without further separation or following additional separation steps. For the former approach, whole blood or blood products were diluted with Hanks Balanced Salt Solution (Invitrogen, Grand Island, NY), pelleted at 1,200 rpm for 30 min, where the supernatant containing anticoagulant was discarded, and the cells were resuspended in culture medium. Alternatively, blood mononuclear cells were isolated by centrifugation over Ficoll-Paque™ (Pharmacia Biotech, Piscataway, NJ) following the manufacturer's protocol. Otherwise, whole blood or blood products were allowed to undergo sedimentation for 3 hours at room temperature or overnight at +4°C, after which plasma and leukocyte layers were carefully aspirated and cells were pelleted. Finally, for some samples, erythrocyte lysis was conducted by mixing up to $1x10^8$ pelleted nucleated cells with 5 ml of ammonium chloride buffer: 144 mM ammonium chloride (Sigma, St. Louis, MO), 17 mM Tris

(ICN Biomedicals, Aurora, OH), pH 7.2, for 5 to 10 min at 37°C with slow rotation, followed by adding equal volume of αMEM (Invitrogen) and centrifugation.

Blood cell cultures

Blood cells were plated at 0.1 to $6.5x10^6$ nucleated cells per cm^2 into T-75, T-162, or T-175 flasks, or 60-mm, 100-mm, or 150-mm dishes (BD Labware, Lincoln Park, NJ; Corning Inc., Corning, NY; Nalge Nunc Intl., Naperville, IL). Alongside with regular tissue culture vessels, those with modified surface (Primaria™, BD Labware), or industrially pre-coated with either human fibronectin or rat collagen type I (BD Labware), were used. Alternatively, the vessels were coated with either 0.1% porcine gelatin (Sigma) or 50 μg/ml human fibronectin (Roche, Mannheim, Germany) for 1 to 5 hours before cell plating. Most cultures were prepared in the medium consisting of αMEM, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Invitrogen), and 20% fetal bovine serum (FBS, Equitech-Bio, Inc., Kerrville, TX) (Medium 1), either with or without 10^{-8} M dexamethasone (Sigma), 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate (AscP, Wako, Osaka, Japan), 10^{-4}M 2-mercapthoethanol (Invitrogen), and 10% human serum (Pel-Freez Biologicals, Rogers, AR). For additional cultures, several other medium formulations were used: 2) X vivo 15, a serum-free medium for lymphocyte growth (Cambrex BioScience, Walkersville, MD); 3) medium designed for embryonic stem cells: DMEM (Invitrogen), L-glutamine, penicillin, streptomycin sulfate, 0.1 M non-essential amino acids (Invitrogen), 10^{-4}M 2-mercapthoethanol, 1,000 U/ml recombinant human Leukemia Inhibitory Factor (R&D Systems, Inc., Minneapolis, MN), 20% FBS; 4) medium formulated for neuronal cells: neurobasal medium (Invitrogen), L-glutamine, penicillin, streptomycin sulfate, 20% FBS, supplement N-2 or G-5 (Invitrogen); 5) medium designed for human "mesodermal progenitor cells":[20] 58% low glucose DMEM, 40% MCDB-201 (Sigma), 2% FBS, 1% ITS$^+$(BD Biosciences, Bedford, MA), penicillin, streptomycin sulfate, 10^{-9}M dexamethasone, 10^{-4} M AscP, 10 ng/ml EGF, and 10 ng/ml PDGF-BB (Sigma). The media were replaced 1 to 3 days after plating and once a week thereafter. To some cultures, guinea pig bone marrow cells irradiated with 6,000 R were added as feeder after the first medium replacement at $2.8\text{-}3x10^7$ nucleated cells per T-75 flask.[21] The cultures were thoroughly inspected under the inverted microscope twice a week. If no adherent connective tissue colonies were found, the cultures were followed for 9 to 25 weeks, fixed with absolute methanol, stained with saturated water solution of methyl violet (Sigma), and yet again assessed for adherent colony formation.

Guinea pig model

All procedures were performed in accordance to an NIDCR approved small animal protocol (97-024).

Blood collection

The blood from adult 5 - 8 month old Hartley male guinea pigs was collected by either cardiac puncture or cannulation of either inferior vena cava or aortic root. The first portion (approximately 0.5 ml) of blood obtained by cannulation was discarded. The procedures were performed under the anesthesia achieved by intraperitoneal injection of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) at 140 mg/kg body weight and Xylazine (Butler, Columbus, OH) at 7 mg/kg body weight. Blood was collected into sterile syringes or tubes containing sodium heparin (Fisher Scientific, Fair Lawn, NJ) at a final concentration of 100 U/ml.

Blood cell cultures

Freshly obtained whole blood was plated into T-75 flasks or 150-mm dishes (BD Labware) at 0.5 – $2.2x10^5$ nucleated cells/cm^2 in culture medium consisting of αMEM, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 20% FBS, 10^{-8} M dexamethasone, and 10^{-4} M AscP. Cells were cultured at 37°C in an atmosphere of 100% humidity and 5% CO_2. Complete medium replacement was performed

one day after plating, at which stage most erythrocytes were removed, and twice a week thereafter. Adherent colonies were released on day 14 to 18 with two consecutive treatments with 1x trypsin-EDTA (Invitrogen). Polyclonal strains were generated by detaching all colonies simultaneously and plating them together into a T-75 flask. Single-colony derived strains were generated in a manner previously described.[22] Each individual colony was separately detached using a polystyrene cloning cylinder (Sigma) and S/P™ High Vacuum Grease (Baxter Healthcare Corp., McGaw Park, IL). Cells were then plated into a single well of a 6-well plate (Nunc, Roskilde, Denmark). Subsequent passages were performed at 70-90%-confluence.

Cultures of guinea pig bone marrow stromal cells (BMSCs)

Bone marrow single cell suspensions were prepared from femora, tibiae, and humeri of Hartley male guinea pigs. Polyclonal BMSC strains were generated as described earlier[21] and used as positive controls in differentiation experiments.

Phenotypic characterization

Polyclonal and single colony-derived strains of blood-derived adherent cells and polyclonal strains of BMSCs were plated in eight-chamber glass slides (Nalge Nunc International) at $0.12x10^5$ cells/cm^2. Histochemical stainings were performed by standard protocols for α-naphthyl acetate esterase, acid phosphatase, and alkaline phosphatase (kits 91-A, 387-A, and 86-C, respectively, Sigma). Immunohistochemical analysis was performed when the cells reached 70% confluence, usually on the next day after plating, with a panel of antibodies (see Table 1) as described previously.[23] Bound antibody was localized by consecutive application of peroxidase-conjugated SuperPicture™ poly HRP conjugate and AEC (red) Single solution (Zymed Labs, Inc., San Francisco, CA). Slides were then counterstained with hematoxylin and mounted in Histomount (Zymed Labs). Slides were viewed using an Axioplan 2 (Carl Zeiss, Inc., Gottingen, Germany), and images were acquired using a DMC-1 digital camera (Polaroid Corp., Cambridge, MA) and formatted using Photoshop 6.0 Software (Adobe Systems, Inc., San Jose, CA).

***In vivo* transplantation assay**

Osteogenic potential of cell strains was analyzed by *in vivo* transplantation assay. Four polyclonal and five single colony-derived strains of guinea pig blood-derived adherent cells (passage 2-6 and 4, respectively), as well as several polyclonal strains of guinea pig BMSCs (passage 2-4) were transplanted, as previously described.[22, 24] Briefly, $1.5\text{-}3.5x10^6$ cells in 1 ml of culture medium were mixed with 40 mg of sterile hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (particle size 0.5-1.0 mm, generously provided by Zimmer, Inc., Warsaw, IN) in a 1.8 ml cryotube (Nunc). After a 90-min incubation at 37ºC with slow rotation (25 rpm), the particles with attached cells were collected by brief centrifugation. A secondary matrix was formed by consecutively mixing the particles with 15 μl of mouse fibrinogen (3.2 mg/ml in PBS) and 15 μl of mouse thrombin (25 U/ml in 2% $CaCl_2$, Sigma) such that a fibrin gel was formed around the particles. The transplants were placed in subcutaneous pockets in the back of 8-15-wk-old immunodeficient female beige mice (bg-nu/nu-xid, Harlan Sprague Dawley, Indianapolis, IN). Three or more transplants per strain were generated, and each experiment was repeated at least twice. The transplants were recovered 8 weeks later, fixed in 4% phosphate-buffered formalin (Sigma), and either embedded in glycol methacrylate or demineralized in 10% EDTA, pH.8.0 (Quality Biological, Inc., Gaithersburg, MD) and embedded in paraffin. Five- or 7-μm sections were prepared and either stained with hematoxylin and eosin (paraffin sections) or Goldner's modified trichrome stain (glycol methacrylate).

***In vitro* pellet culture assay**

Chondrogenic potential of four polyclonal and five single colony-derived strains of guinea pig blood-derived adherent cells (passage 3-5 and 3, respectively) and of polyclonal strains of guinea

pig BMSCs (passage 3) was analyzed using a technique modified from an earlier report.[25] Aliquots of $4x10^5$ cells were pelleted in 15 ml polypropylene conical tubes (Corning) and cultured at 37^{o} C in an atmosphere of 100% humidity and 5% CO_2. Culture medium consisted of DMEM with high glucose (Invitrogen), 1% ITS^{+}, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 10^{-7} M dexamethasone, 50 μg/ml M AscP, 2 mM pyruvate (Invitrogen), and 10 ng/ml human recombinant TGF-β1 (R&D Systems). Medium was replaced every other day. Three or more cultures per strain were generated, and each experiment was repeated at least twice. Cultures were harvested on day 21, fixed in 4% phosphate-buffered formalin, demineralized in 10% EDTA, and embedded in paraffin. Seven μm sections were cut near the middle of the pellets and stained with toluidine blue or immunohistochemically for collagen type II. For the latter staining, HistomouseTM-^{SP} Bulk Kit (Zymed Labs) was used following the manufacturer's recommendations. To facilitate antibody access, sections were predigested with 0.1 U/ml chondroitinase ABC (Seikagaku, Tokyo, Japan) in Tris-acetate buffer, followed by pepsin (Digest-All Kit^{TM}, Zymed Labs). Primary monoclonal antibody against collagen type II (clone 6B3, Chemicon Intl, Temecula, CA) was followed consecutively by a biotinylated secondary antibody, streptavidin-peroxidase, and hydrogen peroxide/chromogen solution (Zymed Labs).

Statistical Analysis

One-way analysis of variance was performed and post-test comparison was done using the Bonferroni multiple comparison test (InStat, GraphPad Software, Inc., San Diego, CA). Differences were considered statistically significant at $p<0.05$.

RESULTS

Human cultures

In cultures of human blood, colonies and fields of adherent polymorphic cells often developed that included a broad array of morphologies, ranging from small round cells and slender spindle-shaped cells ("fibroblastoid cells") to large multi-nuclear cells; these diverse morphological types are shown in Fig. 1 A-C. The abundance of these cells varied from one donor to another and was strongly influenced by the medium composition, with the mixture of dexamethasone and AscP inhibiting their appearance. These cells could persist in primary cultures for many months (Fig. 1 C), but they neither reached confluency nor survived beyond the 1st passage. In previous studies, such cells were shown to exhibit hematopoietic markers, to form osteoclast-like cells, and to represent monocytes/macrophages.[26-28] Contrary to this pattern, adherent colonies consisting of uniform fibroblast-like cells appeared in only four cultures (Fig. 1 D, E). The colonies grew rapidly at the periphery and formed dense central areas. Using a diversified group of blood donors, a number of cell separation techniques, several culture substrata, and a variety of culture media, only 3 donors were identified among 66 studied whose blood yielded one or two adherent connective tissue colony-forming cells; blood from the remaining 63 donors, or 95.5%, demonstrated no such cells. One positive donor (HBl-2) was a healthy 60-year-old male whose blood cells were received as a buffy coat; this donor yielded two colonies, one in a regular flask and another in a fibronectin-coated flask.[14] Another donor (HBl-40) was a healthy 28-year-old male who had undergone G-CSF-induced stem cell mobilization; his blood cells were received as a leukapheresis product; a single colony was formed in a fibronectin-coated flask (Fig. 1D). The third donor (HBl-53) was a 47-year-old female with multiple bone fractures, whose whole blood was received; a single colony was formed in a fibronectin-covered flask (Fig. 1E). All four colonies were formed in cultures of cells plated in Medium 1 with

dexamethasone and AscP, without further cell separation. No adherent connective tissue colonies were formed in cultures from younger donors, as well as from donors suffering from several pathological conditions featuring bone and connective tissue involvement.

From each of the four colonies, a single colony-derived strain of adherent connective tissue cells was subsequently generated by multiple passages. The strains derived from donor HBl-2 were characterized earlier: both of them displayed immunophenotype characteristics of fibroblastic/smooth muscle/weakly osteogenic cells, were capable of adipocytic conversion *in vitro*, while only one of two strains formed bone upon *in vivo* transplantation.[14] We were unable to characterize chondrogenic potential of human single colony-derived strains of circulating adherent connective tissue cells because sufficient numbers of these cells could only be received by passage 3, whilst by this passage, even polyclonal strains of human BMSCs (positive controls) largely lost their ability to form cartilage in preliminary pellet culture experiments.

Guinea pig cultures

Discrete colonies of adherent fibroblast-like cells were reproducibly formed in cultures of guinea pig blood from all animals examined, with no exception. The colony-forming efficiency per $1x10^6$ nucleated cells was 1.1 - 3.9 (mean $\pm$ SEM, 2.7 $\pm$ 0.6) for blood obtained by cardiac puncture and 2.6 - 8.6 (5.3 $\pm$ 1.0) for blood obtained by cannulation ($p > 0.05$). By combining all colonies grown in a flask, four polyclonal (multicolony-derived) strains were established from 4 guinea pigs. When individual colonies were detached and propagated separately, twenty-two clonal (single colony-derived) strains were generated from 3 other animals.

Characterization of polyclonal strains

Each of the polyclonal strains was capable of prolonged proliferation through six or more passages, and generated millions of cells. The strains were comprised of uniform spindle-shaped fibroblastic cells (Fig. 2A), staining variably (from negative to strongly positive) for α-Naphtyl acetate esterase (Fig. 2B), positive for acid phosphatase (Fig. 2C), and negative for alkaline phosphatase (not shown). Immunophenotypically, all four strains were similar to each other and to guinea pig BMSCs: variably positive for markers associated with fibroblasts (fibronectin, collagens type I and III), osteogenic cells (osteonectin, osteopontin, bone sialoprotein), and smooth muscle cells, and negative for markers of hematopoietic cells, macrophages, and endothelial cells (Table 1).

In vivo transplantation assay

Eight weeks post-transplantation, polyclonal strain #1 formed very scant bone limited to a few scattered trabeculae (Fig. 2D), while polyclonal strains #2 and #3 formed fibrous tissue surrounding the ceramic particles with no evidence of bone (Fig. 2E and 2F). Polyclonal strain #4 formed abundant bone (Fig. 2G) comparable in volume with bone developed in the transplants of guinea pig BMSCs (not shown). Bone formed by strain #4 had lamellar-like, well-mineralized structure, was deposited on the surfaces of ceramic particles by mature osteoblasts, and contained embedded osteocytes (Fig. 2H-J). In transplants with large amounts of bone, adipocytes and hematopoietically active marrow including megakaryocytes could be observed in close proximity to the bone surfaces (Fig. 2J). Earlier, it was demonstrated by *in situ* hybridization using guinea pig-specific DNA sequence that bone formed in such transplants was of donor origin.[14]

Pellet culture assay

By day 21, pellet cultures of polyclonal strain #4 dramatically increased in size and developed chondroid morphology with cells encased in chondrocytic lacunae that were intermittently aligned in columnar patterns. The lacunae were surrounded by substantial amounts of matrix that demonstrated robust metachromatic staining, indicating the abundance of sulfated proteoglycans, and strong uniform deposition of

collagen type II protein (Fig. 3D, E). Cultures of polyclonal strains #1, #2, and #3 showed neither a size increase, metachromasia, nor chondroid morphology (Fig. 3A-C). Under identical conditions, guinea pig BMSCs developed cultures similar to those of strain #4 (Fig. 3F). These findings indicate that polyclonal strain #4 and BMSCs, but not the three other polyclonal strains of guinea pig blood-derived adherent cells, demonstrated the capacity to undergo chondrogenic differentiation *in vitro*.

Isolation of single colony-derived strains

Adherent colonies formed in cultures of guinea pig blood were of three morphological types: spindle-shaped, fibroblast-like; flattened, fibroblast-like; and polygonal (Fig. 4A-C). The proportion of colonies of each type varied in cultures from individual animals where spindle-shaped colonies always constituted the most prevalent morphology. In this study, 30 large colonies from three guinea pigs, representing all morphological types, were chosen for further propagation. Twenty-two of these colonies exhibited proliferation in the 1st passage cultures: 6 spindle-shaped, 4 flattened, 11 polygonal, and 1 intermediate. However, only 9 single colony-derived strains from two animals continued proliferation and reached a cell density sufficient to perform the 2nd passage: 5 spindle-shaped and 4 polygonal. At this passage, each strain generated from $0.15x10^6$ to $0.86x10^6$ cells. Only 5 of the 9 strains had a high enough proliferation potential necessary to attain near confluency in the 2nd passage cultures, completing over 20 population doublings and generating several million cells required for two differentiation assays and for immunostaining. All of these 5 strains were of the spindle-shaped type: #10 ($4.0x10^6$ cells at the 3rd passage) and #11 ($17.6x10^6$), both from arterial blood of one animal; #21($9.5x10^6$) and #23 ($3.3x10^6$), both from venous blood of the same animal; #26 ($2.7x10^6$), from venous blood of another guinea pig.

***In vivo* transplantation and pellet culture assays of single colony-derived strains**

Contrary to two polyclonal strains, none of the five single colony-derived strains formed any bone eight weeks after *in vivo* transplantation. The transplants of all strains uniformly revealed fibrous tissue adjacent to ceramic particles (not shown). After 21 days in pellet cultures, two strains, #10 and #11, demonstrated chondrogenesis (Fig. 4D,E) similar to that observed in cultures of polyclonal strain #4 and of guinea pig BMSCs. Conversely, three other strains, #21, #23, and #26, showed no signs of chondrogenic differentiation (Fig. 4F-H).

Characterization of single colony-derived strains

Immunophenotypically, the five strains shared with BMSCs variably positive staining for markers associated with fibroblasts (fibronectin, collagens type I and III), osteogenic cells (osteonectin, osteopontin), and smooth muscle cells (α-smooth muscle actin) (Table 1, Fig. 5A-F). Interestingly, for most of the markers, the degree of variability between the single colony-derived strains was higher than between polyclonal strains of blood-derived cells (Table 1). While all five single colony-derived strains were negative for collagen type II (Table 1), two chondrogenic strains, #10 and #11, contained low numbers of cells strongly to moderately positive for another chondrocytic marker, aggrecan (Fig. 5G,H, Table 1). In this respect, these two blood-derived strains were similar to guinea pig BMSCs (Fig. 5L) and opposite to three non-chondrogenic blood-derived strains, #21, #23, and #26, in which aggrecan-positive cells were totally absent (Fig. 5I-K, Table 1).

DISCUSSION

Adherent connective tissue clonogenic cells can be found reproducibly and at a relatively high concentration in blood from every adult guinea pig. In this study, starting with individual adherent colonies, both polyclonal and single colony-derived strains of guinea pig circulating cells were generated and analyzed. Out of five single colony-derived strains with high proliferation potential, none displayed osteogenic differentiation, while two strains demonstrated chondrogenic differentiation confirmed by established morphological and biochemical criteria. The circulating clonogenic cells that initiated the two strains were therefore capable of both extensive proliferation and differentiation towards cartilage, but not towards bone. These findings may be compared to the differentiation potential previously described for BMSC clones. When mouse and rabbit clones were transplanted *in vivo* within diffusion chambers, approximately one third formed bone and cartilage, while the remaining clones only formed fibrous tissue.[29, 30] By *in vitro* assay, a number of human clones were restricted to osteogenic differentiation, where most clones exhibited bi-potential (osteo-adipo or osteo-chondro), with a smaller proportion demonstrating a capacity for multi-potential (osteo-chondro-adipo).[31, 32] Thus, no pure chondrogenic clones have ever been reported, either *in vivo* or *in vitro*, among BMSCs from three different species, where all clones capable of chondrogenic differentiation were also found to undergo osteogenic differentiation. Osteogenesis has been considered a default pathway for multipotential BMSCs, all of which are reversibly committed to osteogenesis and yet capable of entering other differentiation pathways.[33, 34] Our findings represent, for the first time, the existence of precursor cells with chondrogenic but not osteogenic potential. Apparently, in contrast to BMSCs, at least some guinea pig blood-derived, clonogenic, adherent cells are chondrogenic but lack osteogenic potential. Therefore, within the scope of the conventional (orthodox) BMSC differentiation repertoire[34], these cells can be considered committed chondrogenic precursors.

Out of four polyclonal strains of guinea pig circulating adherent cells, one strain proved to be both osteogenic and chondrogenic. The extent of both bone and cartilage formed by the strain was comparable to that achieved by guinea pig BMSCs under similar conditions. Furthermore, this strain demonstrated the capacity to develop into myelosupportive reticular stroma with associated adipocytes within the transplants. These results imply that some highly proliferative guinea pig blood-derived adherent strains may contain multipotential skeletal stem cells, with the ability for generating at least four stromal cell lineages: osteoblasts/osteocytes, chondrocytes, adipocytes, and reticular cells. While it can not be excluded that polyclonal strains with broad differentiation potential are mixtures of several strains of committed precursors, it seems more likely that such strains indeed represent progeny of circulating multipotential skeletal stem cells.

The four polyclonal strains of guinea pig blood-derived adherent cells invariably consisted of uniform, spindle-shaped, fibroblastic cells. Immunophenotypically, the strains were similar to each other and to guinea pig BMSCs, all sharing a fibroblastic/osteogenic/smooth muscle, non-hematopoietic, non-endothelial signature. The expression of osteogenic markers, such as alkaline phosphatase, osteonectin, osteopontin, and bone sialoprotein, was similar in all polyclonal strains studied and not predictive of their *in vivo* osteogenic potential. Earlier, analysis of monolayer cultures of polyclonal strains of human BMSCs also revealed that the levels of procollagen type I, alkaline phosphatase, and osteopontin were not predictive of osteogenic potential of the strains,[35] whereas the levels of osteopontin and bone sialoprotein mRNA could predict the ability to form bone *in vivo*.[36]

In contrast to the uniform morphology of polyclonal strains of guinea pig circulating cells, individual blood-derived adherent colonies demonstrated three distinct morphological types, consisting of either spindle-shaped, flattened, or polygonal cells. Previously, adherent colonies derived from mouse and rabbit blood were also found to display three morphological types analogous to those described here for guinea pig cultures.[14] Despite pronounced morphological differences, all colony types from all species examined shared similar immunostaining patterns: positive for markers associated with fibroblastic, osteogenic, and smooth muscle cells, and negative for endothelial, hematopoietic, and monocyte/macrophage markers.[14] In this study, among guinea pig colonies of various morphologies, only certain colonies composed of spindle-shaped cells were able to proliferate extensively. The proliferative advantage of spindle-shaped colonies may explain why polyclonal strains generated by combining and expanding colonies of all types uniformly displayed spindle-shaped morphology. Immunophenotypically, single colony-derived strains of spindle-shaped cells demonstrated a higher degree of variability than polyclonal strains. Most importantly, the two single colony-derived strains possessing chondrogenic potential, similarly to highly chondrogenic guinea pig BMSCs, included cells strongly to moderately positive for aggrecan, while three non-chondrogenic strains had no such cells. Likewise, chondrogenic polyclonal strains of human BMSCs were shown to constitutively express the aggrecan message.[37] Aggrecan is the major cartilage proteoglycan, deposition of which is considered a hallmark of chondrogenesis.[38] It may be hypothesized that under non-chondrogenic conditions, a small fraction of chondrogenic cell populations expresses aggrecan constitutively, and thus the expression of aggrecan in monolayer cultures may be predictive of chondrogenic potential.

In this study, the differentiation potential of adherent strains of circulating guinea pig cells was examined using *in vivo* transplantation assay and *in vitro* pellet culture assay. The use of transplantation assays under defined experimental conditions[22, 24, 39, 40] has become a valuable standard for delineating cells' osteogenic potential: not only does it analyze the osteogenic function *in vivo*, it also provides the most convincing result, the formation of histologically proven bone tissue.[34] The pellet (micromass) culture provides an assay of similar significance for chondrogenesis: culturing cells in artificially condensed conditions and inducing them with a chondrogenic cytokine results in the generation of a three-dimensional structure reminiscent of true hyaline cartilage.[25, 41] These two assays are more physiologically relevant and provide more convincing results than widely used monolayer culture differentiation assays.

When studying connective tissue transformation of blood cells, the major concern has been the possibility of contaminating blood samples with cells from the blood vessel or heart walls.[10, 13, 42-45] An important though not definitive result was demonstration that the concentration of adherent colony-forming cells in guinea pig blood was independent of the number of cardiac punctures used to obtain the blood.[46, 47] The cannulation technique has been widely accepted as the safest way of avoiding contamination, even more so when accompanied by discarding the first portion of blood.[13, 42, 45] In the present study, all recommended precautions were taken and yet the concentration of adherent colony-forming cells in guinea pig blood obtained by cannulation was not lower than in blood obtained by cardiac puncture. Our result lends additional support to the view that adherent colony-forming cells do not come from the walls of either heart or blood vessels but are genuinely present in circulation.

Contrary to guinea pigs, our data demonstrate that circulating adherent connective tissue precursors are totally absent in the great majority of human donors, adults, adolescents, and children likewise, normal as well as pathological, and are extremely scarce in a few

donors, in agreement with earlier observations.[2, 14-17] The finding of such precursors in only 3 donors out of 66 may suggest that in postnatal humans, these cells enter the circulation only in very rare, special circumstances, such as following bone fractures or G-CSF-mediated hematopoietic stem cell mobilization. Contrary to this, circulating adherent connective tissue precursors, some of them demonstrating multilineage differentiation potential, were shown to be more prevalent in preterm fetuses and in full term umbilical cord blood,[26, 28, 48-57] although the latter finding has been challenged.[27, 28, 58, 59] Additionally, circulating non-adherent osteogenic precursors were recently found in postnatal humans.[60, 61] If these results are further substantiated, with both rigorous characterization of the osteogenic cells and corroboration of donor origin of bone formed upon their *in vivo* transplantation, the relationship between circulating adherent and non-adherent connective tissue precursors will be of significant interest.

Our results are the first indication that cells with chondrogenic potential can circulate in postnatal animals. Whilst the origin of circulating chondrogenic precursors, their destination in the bloodstream, and their functional role remain unknown, the same holds true for circulating connective tissue precursors in general. It was suggested that the fate of circulating connective tissue precursors may be defined on their arrival to a local tissue by its specific cues,[62] implying that all such precursors are multipotential. According to our data, at least some blood-derived connective tissue precursors are already committed while in circulation. If such circulating chondrogenic precursors can home to cartilage they might participate in osteochondral regeneration contributing to the high ability of articular cartilage of some mammals to self-repair.

Previous studies have demonstrated that the concentration of adherent colony-forming cells varies in different parts of guinea pig circulation, where it appears to be highest in the inferior vena cava, intermediate in heart, and lowest in renal vein.[8] In our study, chondrogenic and non-chondrogenic clonogenic precursors were found in arterial and venous guinea pig blood, respectively. The limited number of analyzed strains precludes us from drawing precise conclusions based on this observation. However, since human blood is generally drawn from veins, this could diminish even further the chances, already very low, of finding chondrogenic precursor cells in human blood. Nevertheless, the search for these elusive cells in postnatal human circulation needs to be continued, in order to determine their role in physiological chondrogenesis and their possible application for the treatment of osteoarthritis.

CONCLUSION

This study demonstrates that adherent, clonogenic, connective tissue cells can be found in blood from only very few postnatal human donors, and even in them, with extremely low frequency. In adult guinea pig blood where such cells are much more common, both committed chondrogenic precursors and multipotential skeletal stem cells have been identified and characterized. These data represent a step towards our understanding of circulating skeletal precursors and, more generally, of circulating connective tissue precursors.

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REFERENCES

1. Chrustschoff GK. Cytological investigations on cultures of normal human blood. J Genet. 1935;31:243-261.
2. Ojeda-Uribe M, Brunot A, Lenat A, et al. Failure to detect spindle-shaped fibroblastoid cell progenitors in PBPC collections. Acta Haematol. 1993;90(3):139-143.
3. Bringuier AF, Seebold-Choqueux C, Moricard Y, et al. T-lymphocyte control of HLA-DR blood monocyte differentiation into neo-fibroblasts. Further evidence of pluripotential secreting functions of HLA-DR monocytes, involving not only collagen but also uromodulin, amyloid-beta peptide, alpha-fetoprotein and carcinoembryonic antigen. Biomed Pharmacother. 1992;46(2-3):91-108.
4. Bucala R, Spiegel LA, Chesney J, et al. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med. 1994;1(1):71-81.
5. Chesney J, Bacher M, Bender A, et al. The peripheral blood fibrocyte is a potent antigen-presenting cell capable of priming naive T cells in situ. Proc Natl Acad Sci U S A. 1997;94(12):6307-6312.
6. Labat ML, Bringuier AF, Seebold-Choqueux C, et al. Possible monocytic origin of chondrosarcoma: in vitro transdifferentiation of HLA-DR blood monocyte-like cells from a patient with chondrosarcoma, into neo-fibroblasts and chondrocyte-like cells. Biomed Pharmacother. 1997;51(2):79-93.
7. Zvaifler NJ, Marinova-Mutafchieva L, Adams G, et al. Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res. 2000;2(6):477-488.
8. Luria EA, Friedenstein AJ, Grosheva AG, et al. Colony-forming precursors of circulating blood fibroblasts. Bull Exper Biol Med. 1989;108:1769-1771.
9. Paul J. Establishment of permanent cell strains from human adult peripheral blood. Nature. 1958;182:808.
10. Hjortdal O, Rasmussen P. In vivo culture of blood cells. I.Fibrogenetic function of some cells in blood clots, as studied by diffusion chamber implants in the peritoneal cavity of rats. Acta Anat. 1969;72:304-319.
11. Petrakis NL. In vivo cultivation of leukocytes in diffusion chambers: requirement of ascorbic acid for differentiation of mononuclear leukocytes to fibroblasts. Blood. 1961;18:310-316.
12. Petrakis NL, Davis M, Lucia SP. The in vivo differentiation of human leukocytes into histiocytes, fibroblasts and fat cells in subcutaneous diffusion chambers. Blood. 1961;17:109-118.
13. Stirling GA, Kakkar VV. Cells in the circulating blood capable of producing connective tissue. Br J Exp Pathol. 1969;50(1):51-55.
14. Kuznetsov SA, Mankani MH, Gronthos S, et al. Circulating skeletal stem cells. J Cell Biol. May 28 2001;153(5):1133-1140.
15. Da WM, Ma DD, Biggs JC. Studies of hemopoietic stromal fibroblastic colonies in patients undergoing bone marrow transplantation. Exp Hematol. 1986;14(4):266-270.
16. Lazarus HM, Haynesworth SE, Gerson SL, et al. Human bone marrow-derived mesenchymal (stromal) progenitor cells (MPCs) cannot be recovered from peripheral blood progenitor cell collections. J Hematother. 1997;6(5):447-455.
17. Wlodarski KH, Janowska-Wieczorek A. Precursor cells of fibroblast colony forming unit in the normal and pathologic human bone marrow and in the peripheral blood. Folia Haematol Int Mag Klin Morphol Blutforsch. 1984;111(5):686-691.
18. Piersma AH, Ploemacher RE, Brockbank KG, et al. Migration of fibroblastoid stromal cells in murine blood. Cell Tissue Kinet. 1985;18(6):589-595.
19. Friedenstein AJ. Precursor cells of mechanocytes. Int Rev Cytol. 1976;47:327-359.
20. Reyes M, Lund T, Lenvik T, et al. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood. Nov 1 2001;98(9):2615-2625.
21. Kuznetsov S, Gehron Robey P. Species differences in growth requirements for bone marrow stromal fibroblast colony formation In vitro. Calcif Tissue Int. 1996;59(4):265-270.
22. Kuznetsov SA, Krebsbach PH, Satomura K, et al. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res. 1997;12(9):1335-1347.
23. Gronthos S, Mankani M, Brahim J, et al. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000;97(25):13625-13630.
24. Krebsbach PH, Kuznetsov SA, Satomura K, et al. Bone formation in vivo: comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. Transplantation. 1997;63(8):1059-1069.
25. Johnstone B, Hering TM, Caplan AI, et al. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res. Jan 10 1998;238(1):265-272.
26. Bieback K, Kern S, Kluter H, et al. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells. 2004;22(4):625-634.
27. Wexler SA, Donaldson C, Denning-Kendall P, et al. Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. Br J Haematol. Apr 2003;121(2):368-374.

28. Yu M, Xiao Z, Shen L, et al. Mid-trimester fetal blood-derived adherent cells share characteristics similar to mesenchymal stem cells but full-term umbilical cord blood does not. Br J Haematol. Mar 2004;124(5):666-675.
29. Bennett JH, Joyner CJ, Triffitt JT, et al. Adipocytic cells cultured from marrow have osteogenic potential. J Cell Sci. May 1991;99 (Pt 1):131-139.
30. Gerasimov Iu V, Fridenshtein A, Chailakhian RK, et al. [Differentiation potentials of clonal strains of bone marrow fibroblasts]. Biull Eksp Biol Med. Jun 1986;101(6):717-719.
31. Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci. Apr 2000;113 (Pt 7):1161-1166.
32. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science. Apr 2 1999;284(5411):143-147.
33. Bianco P, Gehron Robey P. Marrow stromal stem cells. J Clin Invest. Jun 2000;105(12):1663-1668.
34. Bianco P, Riminucci M, Gronthos S, et al. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 2001;19(3):180-192.
35. Mendes SC, Tibbe JM, Veenhof M, et al. Relation between in vitro and in vivo osteogenic potential of cultured human bone marrow stromal cells. J Mater Sci Mater Med. Oct 2004;15(10):1123-1128.
36. Jaquiery C, Schaeren S, Farhadi J, et al. In vitro osteogenic differentiation and in vivo bone-forming capacity of human isogenic jaw periosteal cells and bone marrow stromal cells. Ann Surg. Dec 2005;242(6):859-867, discussion 867-858.
37. Mwale F, Stachura D, Roughley P, et al. Limitations of using aggrecan and type X collagen as markers of chondrogenesis in mesenchymal stem cell differentiation. J Orthop Res. Aug 2006;24(8):1791-1798.
38. Knudson CB, Knudson W. Cartilage proteoglycans. Semin Cell Dev Biol. Apr 2001;12(2):69-78.
39. Friedenstein AJ, Chailakhyan RK, Gerasimov UV. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet. May 1987;20(3):263-272.
40. Mankani MH, Kuznetsov SA, Shannon B, et al. Canine cranial reconstruction using autologous bone marrow stromal cells. Am J Pathol. Feb 2006;168(2):542-550.
41. Muraglia A, Corsi A, Riminucci M, et al. Formation of a chondro-osseous rudiment in micromass cultures of human bone-marrow stromal cells. J Cell Sci. Jul 15 2003;116(Pt 14):2949-2955.
42. Allgower M, Hulliger L. Origin of fibroblasts from mononuclear blood cells: a study on in vitro formation of the collagen precursor, hydroxyproline, in buffy coat cultures. Surgery. 1960;47:603-610.
43. Rangan SR. Origin of the fibroblastic growths in chicken buffy coat macrophage cultures. Exp Cell Res. 1967;46(3):477-487.
44. Rasmussen P, Hjortdal O. In vivo culture of blood cells. II. The origin of fibroblasts in blood and blood buffy coat cultures studied by diffusion chamber implants in the peritoneal cavity of rats. Acta Anat (Basel). 1969;72(3):476-486.
45. Ross R, Lillywhite JW. The fate of buffy coat cells grown in subcutaneously implanted diffusion chambers. A light and electron microscopic study. Lab Invest. 1965;14(9):1568-1585.
46. Luria EA, Panasyuk AF, Friedenstein AJ. Fibroblast colony formation from monolayer cultures of blood cells. Transfusion. 1971;11(6):345-349.
47. Panasyuk AF, Luria EA. Formation of colonies of fibroblasts in cultures of circulating blood cells. Bull Exper Biol Med. 1970;70:1319-1321.
48. Campagnoli C, Roberts IA, Kumar S, et al. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood. Oct 15 2001;98(8):2396-2402.
49. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol. 2000;109(1):235-242.
50. Prindull G, Ben-Ishay Z, Ebell W, et al. CFU-F circulating in cord blood. Blut. 1987;54(6):351-359.
51. Rosada C, Justesen J, Melsvik D, et al. The human umbilical cord blood: a potential source for osteoblast progenitor cells. Calcif Tissue Int. Feb 2003;72(2):135-142.
52. Lee OK, Kuo TK, Chen WM, et al. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood. Mar 1 2004;103(5):1669-1675.
53. Yang SE, Ha CW, Jung M, et al. Mesenchymal stem/progenitor cells developed in cultures from UC blood. Cytotherapy. 2004;6(5):476-486.
54. Gang EJ, Hong SH, Jeong JA, et al. In vitro mesengenic potential of human umbilical cord blood-derived mesenchymal stem cells. Biochem Biophys Res Commun. Aug 13 2004;321(1):102-108.
55. Goodwin HS, Bicknese AR, Chien SN, et al. Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. Biol Blood Marrow Transplant. 2001;7(11):581-588.
56. Ye ZQ, Burkholder JK, Qiu P, et al. Establishment of an adherent cell feeder layer from human umbilical cord blood for support of long-term hematopoietic progenitor cell growth. Proc Natl Acad Sci U S A. 1994;91(25):12140-12144.
57. Kogler G, Sensken S, Airey JA, et al. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med. Jul 19 2004;200(2):123-135.
58. Gutierrez-Rodriguez M, Reyes-Maldonado E, Mayani H. Characterization of the adherent cells developed in Dexter-type long-term cultures from human umbilical cord blood. Stem Cells. 2000;18(1):46-52.
59. Mareschi K, Biasin E, Piacibello W, et al. Isolation of human mesenchymal stem cells: bone marrow versus

umbilical cord blood. Haematologica. Oct 2001;86(10):1099-1100.
60. Eghbali-Fatourechi GZ, Lamsam J, Fraser D, et al. Circulating osteoblast-lineage cells in humans. N Engl J Med. May 12 2005;352(19):1959-1966.
61. Khosla S, Eghbali-Fatourechi GZ. Circulating cells with osteogenic potential. Ann N Y Acad Sci. Apr 2006;1068:489-497.
62. Canalis E. The fate of circulating osteoblasts. N Engl J Med. May 12 2005;352(19):2014-2016.
63. Fisher LW, Stubbs JT, 3rd, Young MF. Antisera and cDNA probes to human and certain animal model bone matrix noncollagenous proteins. Acta Orthop Scand Suppl. 1995;266:61-65.

Figure 1. Adherent cell growth in primary cultures of human peripheral blood cells. A-C, non-fibroblastic cells (macrophages and their derivatives); D-E, colonies of fibroblast-like connective tissue cells. A, B, D, E, live cultures, C, fixed culture stained with methyl violet.

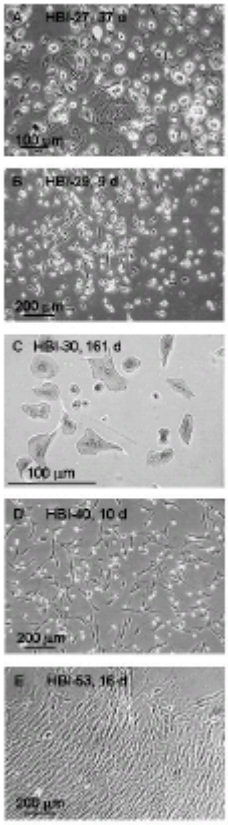

Figure 2. Characterization of polyclonal strains of guinea pig blood-derived adherent cells. A, live confluent culture, unstained; B, C, cultures stained for α-naphthyl acetate esterase (B) and acid phosphatase (C). D-J, eight-week-old transplants of polyclonal strains. New bone (b) was scant in the transplants of strain #1 (D), abundant in the transplants of strain #4 (G), and absent in the transplants of strains #2 (E) and #3 (F) where just fibrous tissue (ft) could be observed surrounding ceramic particles (c). H-J, higher magnification of the transplants of strain #4; H and I represent the same area viewed under conventional (H) and polarized light (I). In I, collagen bundles were organized in parallel patterns consistent with lamellar-like structure of the new bone. D-I, demineralized sections stained with hematoxylin and eosin. J, undemineralized section stained with Goldner's trichrome. Osteoblasts (yellow arrow) deposited unmineralized osteoid (red arrow, red) that, upon mineralization, became bone matrix (b, green). Abundant bone was accompanied by hematopoietic marrow (h) that included adipocytes (a) and megakaryocytes (blue arrow).

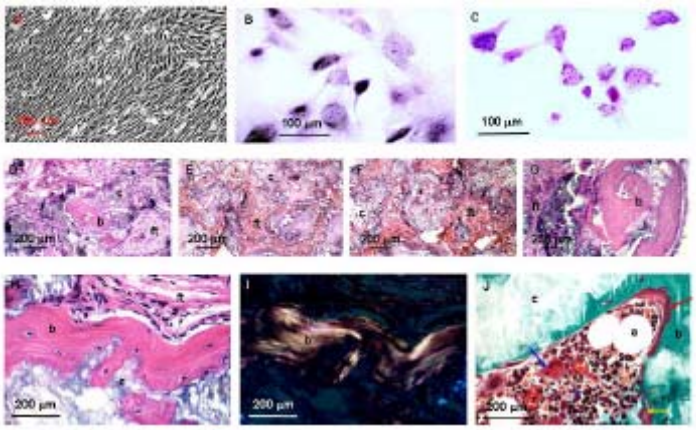

Figure 3. Twenty one-day-old pellet cultures of four polyclonal strains of guinea pig blood-derived adherent cells and of a polyclonal strain of guinea pig BMSCs. Cultures of strains #1 (A), #2 (B), and #3 (C) showed neither size increase nor metachromatic staining. To the contrary, cultures of strain #4 (D, E) and of BMSCs (F) demonstrated drastic increase in size, strong metachromasia with toluidine blue (D, F), cartilage-like morphology, and positive staining for Collagen type II (E). A-D, F, staining with toluidine blue; E, immunostaining for collagen type II.

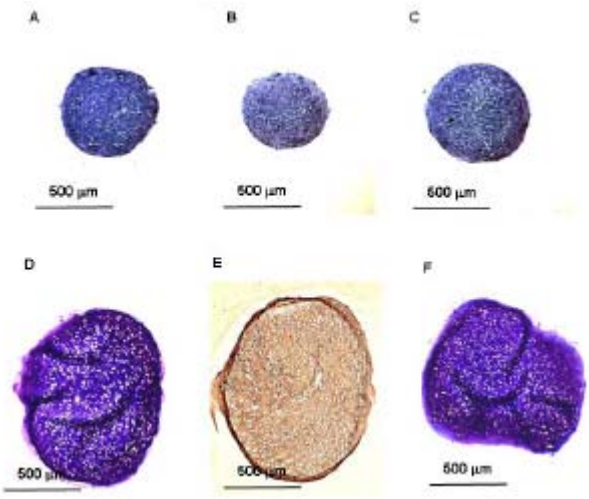

Figure 4. Morphological types of adherent colonies in cultures of guinea pig blood and chondrogenic differentiation of single colony-derived strains of guinea pig circulating adherent cells. A-C, three morphological types of adherent colonies in cultures of guinea pig blood: spindle-shaped, fibroblast-like (A), flattened, fibroblast-like (B), and polygonal (C); live cultures. D-H, twenty one-day-old pellet cultures of five single colony-derived strains of guinea pig circulating adherent cells. Cultures of strains #10 (D) and #11 (E) demonstrated chondrogenic differentiation, while cultures of strains #21 (F), #23 (G), and #26 (H) did not. Staining with toluidine blue.

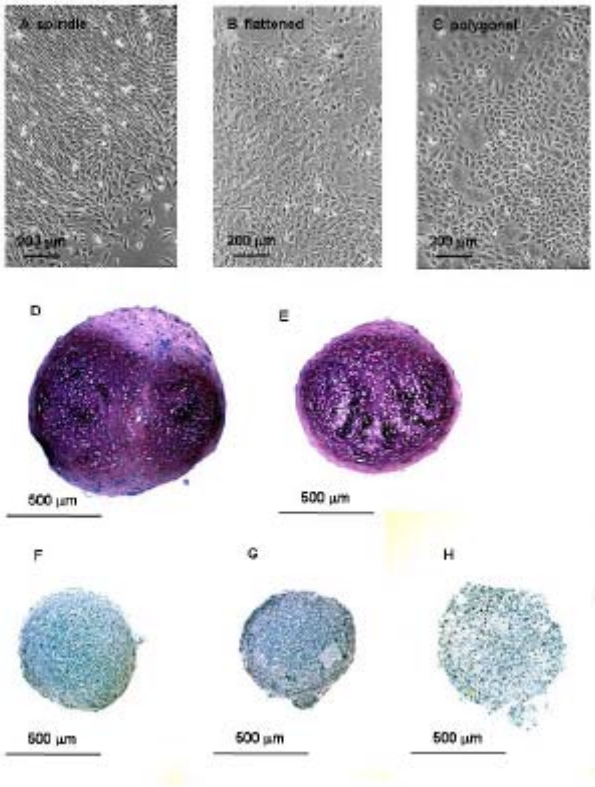

Figure 5. Immunophenotype of single colony-derived strains of guinea pig circulating adherent cells. A-F, staining for osteonectin (A), osteopontin (B), collagen type I (C), collagen type III (D), fibronectin (E), and α-smooth muscle actin (F). G-L, staining for aggrecan: while strains # 10 (G), #11 (H), and guinea pig BMSCs (L) contained a minority of strongly and moderately stained cells, strains #21, #23, and #26 (I-K) were totally negative. Counterstaining with hematoxylin; the 50 μm scale bar applies to all pictures.

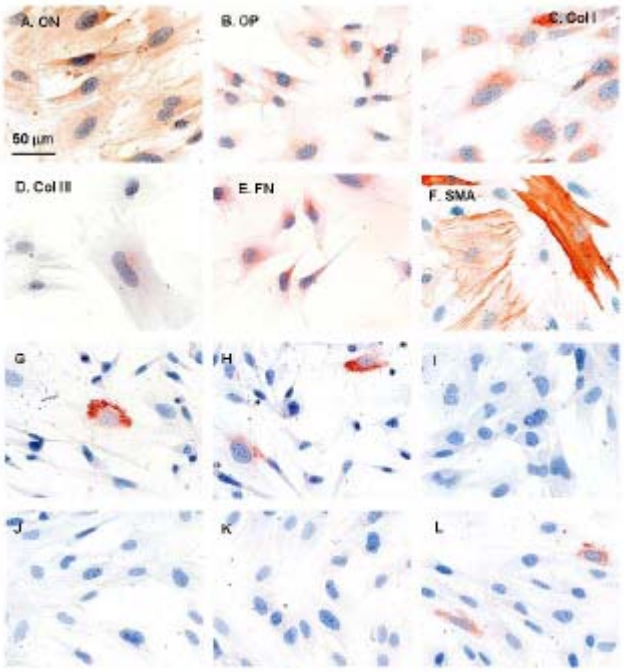

Table 1. Immunophenotypic characterization of polyclonal and single colony-derived strains of guinea pig blood-derived adherent cells and of polyclonal strains of guinea pig bone marrow stromal cells

Marker	Positive in	Polyclonal strains of blood-derived adherent cells				Single colony-derived strains of blood-derived adherent cells					Polyclonal strains of BMSCs
		#1	#2	#3	#4	#10	#11	#21	#23	#28	
Osteonectin (BON-1)[a]	Osteogenic cells	++/+	++/+	+	++/+	++	++	++	++	++/+	++
Osteopontin (LF123)[a]	Osteogenic cells	++/+	++/+	++/+	++/+	++/+	++/+	++/+	+/-	+/-	++/+
Bone Sialoprotein (LF83)[a]	Osteogenic cells	+	++/+	++/+	++/+	ND	ND	ND	ND	ND	++/+
Collagen type I (LF41)[a]	Fibroblasts	++	++	++	++	++	++/+	++	++/+	+/-	++
Collagen type III (LF71)[a]	Fibroblasts	+	++/+	+	+	+/-	+/-	-	-	+/-	+
Fibronectin (MsGP 13; Serotec)	Fibroblasts	++/+	++	++/+	++/+	++	++	++	++	++/+	++
CD45 (1H-1; Serotec)	Leukocytes	-	-	-	-	ND	ND	ND	ND	ND	-
Anti-macrophage (MR-1; Serotec)	Macrophages	-	-	-	-	ND	ND	ND	ND	ND	-
α-Smooth muscle actin (1A4; Dako)	Smooth muscle	++	++	++	++/+	++/+/-	++/+/-	++	++	++/+/-	++/+/-
Anti-smooth muscle (ACL-10002; Accurate Chemical Scientific)	Smooth muscle	++/+	++/+	++/+	+	ND	ND	ND	ND	ND	+
Anti-endothelium (EN4; Accurate Chemical Scientific)	Endothelial cells	-	-	-	-	ND	ND	ND	ND	ND	-
Collagen type II (6B3, Chemicon)	Chondrocytes	ND	ND	ND	ND	-	-	-	-	-	-
Aggrecan (AB1031, Chemicon)	Chondrocytes	ND	ND	ND	ND	++/+/-	++/+/-	-	-	-	++/+/-

++, intensive staining; +, weak staining; -, negative staining; ND, not done

[a] [83], provided by Dr. L. Fisher, National Institute of Dental and Craniofacial Research, National Institutes of Health (Bethesda, MD).

Circulating Connective Tissue Precursors: Extreme Rarity in Humans and Chondrogenic Potential in Guinea Pigs

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